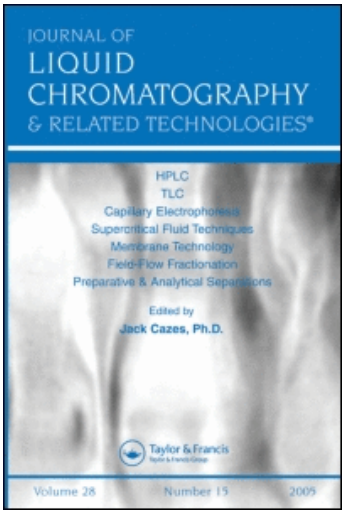

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Determination of Quinic and Shikimic Acids in Products Derived from Bees and their Preparates by HPLC

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Determination of Quinic and Shikimic Acids in Products Derived from Bees and their Preparates by HPLC

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Abstract: An HPLC method was developed for determination of some organic acids in propolis, honey, and pastilles. The on-line coupled Separon SGX C18 and Polymer IEX H-form column with mobile phase composed of sulphuric acid 9 mmol/L and methanol (95:5) at a flow rate 0.8 mL/min and spectrophotometric detection at 215 nm were used for the determination of quinic acid and shikimic acid. Limit of detection of quinic acid was 10 μg/mL and shikimic acid 0.43 μg/mL. Limits of quantitation were 30 μg/mL for quinic acid and 1.26 μg/mL for shikimic acid. Shikimic acid concentrations from 4.2 to 309.0 μg/g and quinic acid concentrations from 0.2 to 6.2 mg/g were determined in all tested bees products. The differences in the acid concentrations were observed for propolis samples from East and West Slovakia.

Keywords: Quinic acid, Shikimic acid, Propolis, Honey, Pastilles, HPLC

INTRODUCTION

The high relevance of organic acids to food technology has fostered the development of a host of methods for their determination (volumetric,

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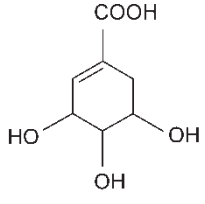
electrochemical, enzymatic, or chromatographic). HPLC has simplified the analysis for various food constituents, including organic acids. It allows the fast, sensitive, and nearly specific determination of organic acids and involves uncomplicated sample treatment.[1]

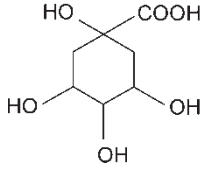
Quinic acid (pK = 3.58) and shikimic acid (pK = 4.76) (Fig. 1) are acyclic monocarboxylic acids. They are intermediate molecules produced in the "shikimate pathway", the biosynthetic pathway of some acid, aromatic aminoacids, flavonoids, and other compounds presented in plants and microorganisms.[2] Quinic and shikimic acid are often found in the free acid form or bound state with one of their hydroxyl functions esterified to a phenolic acid. The quantitative determination of these acids can be done by ion-exclusion and reversed-phase HPLC with refractive index detection or spectophotometric detection,[3–7] capillary zone electrophoretic methods with spectrophotometric detection,[4,8] or gas chromatography with mass spectrometry detection.[9,10]

Propolis is a resinous substance collected by bees from various tree buds (poplar, birch, beech, horse chesnut, alder, and various conifers). The composition of propolis and its properties depends on the local floral and climatic conditions of the collection of resin and secrets by bees.[11–13] Raw propolis is composed of 50% resin, 10% essential oils, 30% wax, 5% pollen, and 5% other organic compounds.[14] Propolis used extensively in folk medicine has been reported to possess various biological activities, such as antibacterial, antifungal, antiviral, anti-inflammatory, local anesthetic, antioxidant, and immunostimulating.[13–17]

Honey is a very important energy food. Honey varies greatly in quality, and its quality is assessed largely on the base of colour, flavour, and density. The quality of honey is judged by its botanical and floral origin and chemical composition. Its composition depends on the types of flowers used by the bees, as well as regional climatic conditions.[18,19] The largest portion of honey composition represent sugars (fructose and glucose are the most abundant sugars



Shikimic acid



Quinic acid

Figure 1. Chemical structures of the studied acids.

found) but others are usually mentioned, namely saccharose, maltose, trehalose, and elizitose. It also has a wide range of minor constituents including polyphenols, enzymes, organic acids, aminoacids, proteins, and so on.[20,21]

The aim of this work was to use the liquid chromatographic method for determination of shikimic and quinic acid in products from bees (propolis and honey) and propolis and honey pastilles.

EXPERIMENTAL

Chemicals and Reagents

The standards of shikimic acid and quinic acid were obtained from ICN Biomedicals (USA).

Acetonitril and methanol for HPLC were obtained from Merck (Germany). Sulphuric acid 96% for analysis was obtained from Mikrochem (Slovakia).

The samples of propolis were collected from the beehive before the winter season and were stored desiccated and in the dark until processing. The work was carried out on three samples of propolis collected in Slovakia. The samples of propolis (I-III) were harvested in the years 2003–2005.

The forest and floral honey samples from Slovakia were used for analysis. The samples were harvested in 2005 and stored in darkness at room temperature until the analysis.

Propolis pastilles and herbal pastilles with honey were obtained from a commercial network.

Sample Preparation

Propolis Samples

I) Propolis from west of Slovakia (2005): Propolis (250 g) was extracted with 500 mL of pure ethanol for three days at temperature 25°C. The extract was filtered and a volume of 20 μL was injected into the liquid chromatograph.

II), III) propolis from east of Slovakia (2004 (II), 2003 (III)): Propolis (150 g) was extracted with 500 mL of pure ethanol for three days at 25°C. The extract was centrifuged at 1000 × g for 10 min and a volume of 20 μL was injected into the liquid chromatograph.

Pastilles

Powdered pastilles (1 g) were extracted with methanol (6 mL) during 1 hour. The extract (1 mL) was dried with a stream of compressed air and diluted in 0.1 mL of methanol. The volume of 20 μL was injected into the liquid chromatograph.

The extraction procedure for propolis samples and pastilles was repeated two times. In all cases the concentration of studied acids in the second extracts was below the limit of detection.

Honey Samples

A 2 g amount of sample was dissolved in 10 mL of distilled water, filtered (0.45 μm filter) and 20 μL of this solution was injected into the analytical column.

Instrumentation

Experiments were conducted on Hewlett Packard (series 1100) HPLC system consisting of a quaternary pump equipped with an injection valve (Rheodyne), diode array detector, and thermostat. Chromatographic columns Symmetry Shield RP18 (150 × 3,9 mm I.D., 5 μm) (Waters, USA), Reprosil 100 C18 (125 × 3 mm I.D., 5 μm) (Watrex, Slovakia), Polymer IEX H-form (250 × 8 mm I.D., 8 μm) (Watrex, Slovakia), and the short column Separon SGX C18 (10 × 4 mm I.D., 7 μm) (Watrex, Slovakia) were tested for separation of acids.

The tested mobile phases for separation of quinic and shikimic acid were mixtures of sulphuric acid (concentration 5–20 mmol/L) and methanol or acetonitrile in different ratios described in Results and Discussion.

All the separations were carried out at a flow rate of 0.8 mL/min and the column temperature was 25°C. For quantitative analysis, UV wavelength at 215 nm was used for the detection. The peak area of related compounds was used for quantitative calculations. The standards were dissolved in water, and filtered with a 0.45 μm filter when necessary.

RESULTS AND DISCUSSION

HPLC Separation

First, suitable chromatographic conditions, stationary phase, mobile phase, and type of detection, were selected.

For the purpose of the study, two C_{18} columns were tested: Reprosil 100 C18 and Symmetry Shield RP18 column. Both C_{18} columns were not suitable for analysis of the studied acids because the values of resolution between quinic acid and shikimic acid in all tested mobile phases were low ($R_s \leq 0.5$).

When the Polymer IEX H-form column was used, the separation mechanism was ion exclusion, where the separation is accomplished by according to differences in pK_a values. The separation was achieved using sulfonated styrene-divinylbenzene sorbent in hydrogen cycle as stationary phase and sulphuric acid-methanol or acetonitrile in the ratio 90:10 and 95:5 (v/v) as mobile

phase. The influence of the concentration of sulphuric acid in the ratio 5–20 mmol/L was also checked. The significant relationship between compounds separation and concentration of sulphuric acid in mobile phase was not observed. By using 9 mmol/L sulphuric acid as mobile phase a shorter analysis time and symmetrical peaks of separated acids were observed. The best conditions for the separation of quinic and shikimic acids were mobile phases composed of methanol and 9 mmol/L sulphuric acis 5:95 (v/v) and flow rate 0.8 mL/min. Although the separation between acids was satisfy ($R_s = 2.2$), both coeluted with the peak presenting in the matrix (depending on the analysed samples). For that reason, the short C_{18} column was put in series with an ionex column to remove interferencies. Under this condition, the separation of quinic and shikimic acids was accomplished in a short time (less than 10 min). The parameters of the system suitability test are shown in Table 1.

Detection

Detection wavelength was chosen according to the absorbance spectra of all separated compounds. Quinic and shikimic acid had a detector response in the range of wavelength from 200 to 240 nm. The wavelength of 215 nm was used for quantitative evaluation. The refractive index detection was also tested for the detection of quinic and shikimic acid. No better results (LOD, LOQ) were achieved.

Figure 2 shows the chromatogram of separation of quinic and shikimic acids using coupled a C_{18} and ionex column and UV spectra of standards obtained by photodiodearray detector.

Method Validation

The system suitability test and validation parameters were examined. The linearity of the analytical method was determined by means of calibration

Table 1. The results of system suitability test for quinic and shikimic acid

Parameter	Quinic acid	Shikimic acid
Repeatability-t_R (%)[a]	1.0	0.9
Repeatability-A (%)[a]	0.53	0.66
Theoretical plates[b]	5187	5262
Resolution[b]	2.21	
Asymmetry[b]	0.94	1.03

[a]Made in six replicates.
[b]Made in three replicates.

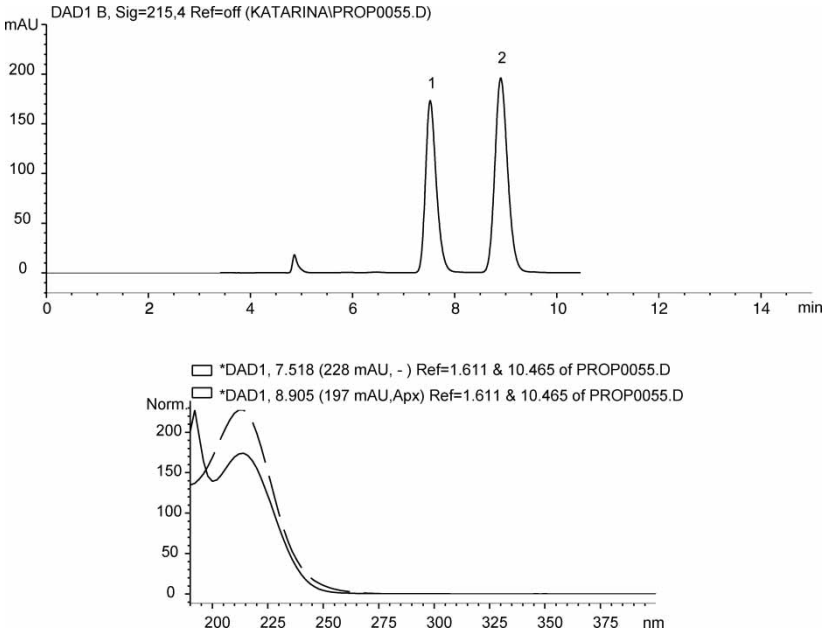


Figure 2. HPLC chromatogram and UV spectra of standard solution of quinic-(1) and shikimic-(2) acid using coupled a C_{18} and ionex column. Chromatographic conditions: Separon SGX C18 and Polymer IEX H-form, mobile phase: 9 mmol/L sulphuric acid - methanol (95:5 v/v), flow rate 0.8 mL/min, UV detection at 215 nm.

curves. For analytes, a regression line was fitted by applying the linear regression model based on the least square method. Based on an eight point calibration, a linear response was observed in concentration range from 1.26 μg/mL to 1.0 mg/mL for shikimic acid and from 30.1 μg/mL to 24.1 mg/mL for quinic acid, with correlation coefficients over 0.999.

The precision was measured for three days using spiked propolis, pastilles, and honey samples for two concentration levels. The evaluation of the method precision was carried out in a day (intraday precision) and in three different days (interday precision) and evaluated by means of the RSD. The accuracy of the method was determined by replicate analysis of samples with standard addition of the analytes. It was tested by using six replicates for two concentration levels of quinic and shikimic acid (0.25 and 2.5 mg/mL for quinic acid, 2.5 and 25 μg/mL for shikimic acid) intra-and interday (in three different days). The results are shown in Table 2. The obtained values for the precision are less than 7% and for the accuracy less than 6% for both analytes.

Limit of detection (LOD) was measured as the lowest amount of the analyte that may be detected to produce a response that is different from that of a blank ($S/N = 3$). The quinic acid concentration obtained was

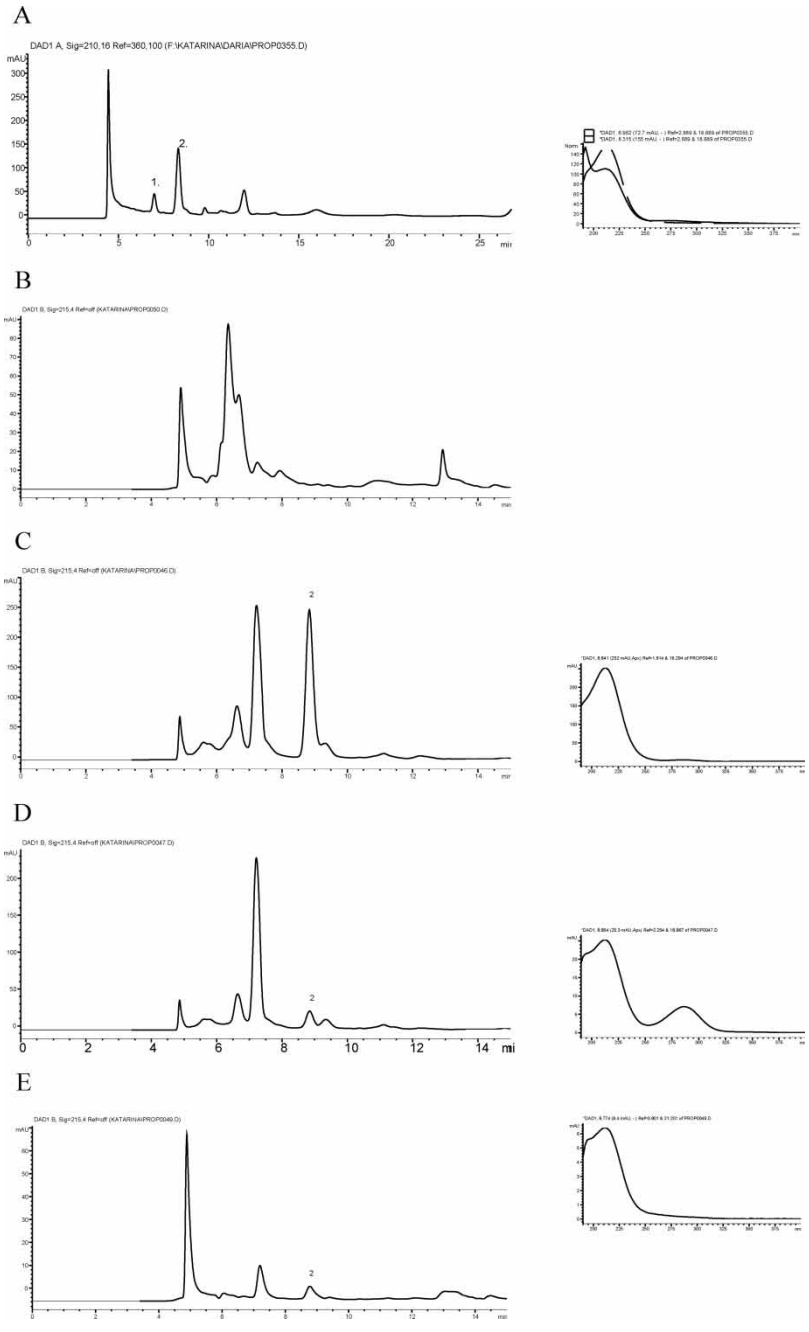


Figure 3. HPLC chromatograms and UV spectra of ethanol extract of propolis (I) (A), propolis pastilles (B), forest (C), and floral (D) honey, and herbal pastilles with honey (E). Chromatographic conditions: Figure 2.

Table 2. The values of precision and accuracy obtained for two concentration levels of quinic and shikimic acid

	Precision (RSD%)		Accuracy (RSD%)	
Concentration	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Quinic acid (mg/mL)				
0.25	4.5	5.8	3.6	4.4
2.5	3.4	5.3	2.7	3.8
Shikimic acid (μg/mL)				
2.5	5.9	7.0	3.9	5.8
25	5.5	6.8	3.1	3.8

Made in three replicates.

10 μg/mL and shikimic acid was 0.43 μg/mL. Limit of quantitation (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise (S/N = 10). The quinic acid concentration obtained was 30.1 μg/mL and shikimic acid 1.26 μg/mL. This concentration corresponds to the first point of the calibration curve.

Analytical Applications

The developed method has been applied to analysis of products derived from bees and their preparates. The three samples of propolis (ethanol extracts), two samples of honey (floral and forest) from Slovakia, and propolis and herbal pastilles with honey were analysed by a two-column (C_{18}–ionex) HPLC method with spectrophotometric detection. The chromatograms of the samples indicate the presence of shikimic acid from 4.2 to 309.0 μg/g, and quinic acid from 0.2 to 6.2 mg/g. The quantitation of the analytes was achieved by the corresponding calibration curve. The assay results are listed

Table 3. Assay results for quinic and shikimic acid in tested samples of propolis, honey and pastilles

Sample	Quinic acid (mg/g)	Shikimic acid (μg/g)
Propolis I	6.2 ± 0.3	309.0 ± 7.5
Propolis II	0.2 ± 0.01	43.3 ± 2.7
Propolis III	<LOD	38.0 ± 2.2
Propolis pastilles	<LOD	<LOD
Forest honey	<LOD	111.6 ± 4.3
Floral honey	<LOD	<LOD
Herbal pastilles with honey	<LOD	0.419 ± 0.1

Made in three replicates.

in Table 3. The concentration of studied acids in tested propolis pastilles was below the detection limit of the used method. In the case of floral honey, the peak of shikimic acid interfered with compound of different UV spectra. The differences in the acids concentration were obtained for propolis samples from east and west of Slovakia. It is probably the effect of different local floral and climatic conditions of the collection of resin and secrets by bees. The chromatograms shown in Fig. 3 illustrate the analysis of some tested bees products.

CONCLUSION

The HPLC-DAD developed method was used for the determination of acids of "shikimate pathway" in bee products and their preparates.

A chromatographic column Separon SGX C18 coupled to a Polymer IEX H-form column using the mobile phase composed of sulphuric acid 9 mmol/L and methanol (95:5) and spectrophotometric detection at 215 nm were used for the determination of quinic acid and shikimic acid in less than 10 min of the total run.

The method has been validated and it has been shown that it is reproducible, linear, precise, and accurate both in upper and lower concentration range. The method was applied for quantification of the quinic acid and shikimic acid in propolis and honey and their preparates without complicated pretreatment.

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